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<p><b>(54) Title:</b> ADULT OLIGODENDROCYTE PRECURSOR CELL COMPOSITIONS AND METHODS</p>		
<p><b>(57) Abstract</b></p> <p>A substantially pure culture of adult oligodendrocyte precursor (progenitor) cells is disclosed. Also disclosed are methods of purifying and culturing/expanding adult oligodendrocyte precursor cells from a suspension of cells, and methods of differentiating perinatal oligodendrocyte precursor cells from oligodendrocyte precursor cells.</p>		

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# ADULT OLIGODENDROCYTE PRECURSOR CELL COMPOSITIONS AND METHODS

## FIELD OF THE INVENTION

- 5           The present invention relates to substantially pure cultures of adult oligodendrocyte precursor cells and methods of isolating, purifying and using such cells.

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#### 15 BACKGROUND OF THE INVENTION

Demyelinating diseases such as Multiple Sclerosis generally destroy oligodendrocytes and their myelin while leaving axons intact. The preservation of axons suggests that considerable repair might be possible if the axons could be remyelinated (*i.e.*, if new oligodendrocytes could be generated).

- 20 Both the rodent and the human adult central nervous system (CNS) seem to have at least some ability to recover from demyelinating lesion by generation of new oligodendrocytes and remyelination (Ludwin, 1981; Prineas and Connell, 1979; Raine *et al.*, 1981; Prineas *et al.*, 1989; Dubois-Dalcq and Armstrong, 1990). The source of the new oligodendrocytes, however, is not clear. The new oligodendrocytes might arise either
- 25 from surviving oligodendrocytes or from oligodendrocyte precursor cells (O-2As) that persist in the adult central nervous system (adult oligodendrocyte precursor cells).

It is not likely that mature oligodendrocytes are a source of new oligodendrocytes, since mature oligodendrocytes appear to be terminally differentiated cells which, like neurons, have little capacity for proliferation (see, for example, Barres and Raff, 1994).

- 30 Further, studies purporting to demonstrate the generation of new oligodendrocytes from mature oligodendrocytes are not convincing because some perinatal oligodendrocyte precursor cells are invariably present in the experimental cultures (Wood and Bunge, 1991).

On the other hand, a number of studies are consistent with a role of adult oligodendrocyte precursor cells in recovery from demyelinating lesions. For example, after

virally-induced demyelination of the mouse spinal cord, a population of cells with the antigenic profile of oligodendrocyte precursor cells incorporated  $^3\text{H}$ -thymidine and increased in number during the recovery phase (Godfraind *et al.*, 1989; Carroll and Jennings, 1994). Furthermore, adult precursors in mixed optic nerve cultures, in which fewer than 5% of the cells are adult precursors, have been demonstrated to generate new oligodendrocytes (French-Constant and Raff, 1986; Wren *et al.*, 1992). In this respect, it has been suggested (French-Constant and Raff, 1986) that adult oligodendrocyte precursor cells may be analogous to the stem cells that persist in adult muscle (Grounds, 1991), having the capacity to revert after injury to rapidly dividing cells in order to quickly supply new oligodendrocytes.

#### SUMMARY OF THE INVENTION

In one aspect, the present invention includes a culture of mammalian cells, where more than about 95% of the cells are adult oligodendrocyte precursor cells. In one embodiment, more than about 99% of the cells are adult oligodendrocyte precursor cells. The cells may be purified from any of a number of central nervous system sources, *e.g.*, rat optic nerve, human biopsy, rat or human temporal lobe, etc. Exemplary sources of the cells include rat and human CNS tissue. The invention also includes an isolated population of adult oligodendrocyte precursor cells, where more than about 95% of the population are adult oligodendrocyte precursor cells. A population of cells typically consists of at least 100 cells.

Also included in the invention is a pharmaceutical composition containing the purified cells described above. Such a pharmaceutical composition is suitable for use in the manufacture of a medicament for treatment of demyelinating diseases (*e.g.*, multiple sclerosis) and other conditions due at least in part to loss of CNS neuron myelination, such as brain trauma (*e.g.*, due to acute injury or infection). A treatment method employing such a medicament includes obtaining a population of purified or substantially-purified adult oligodendrocyte precursor cells from the subject, and implanting or injecting the cells into the diseased or injured brain region, wherein the implanted cells remyelinate demyelinated axons near the site of injections or implantation. In one embodiment, the cells are expanded in culture prior to the implanting or injecting. The composition is also useful in treatment of an individual suffering from symptoms due to a demyelinating disease or condition. The treatment includes delivering the composition to a demyelinated region of the individual's central nervous system, wherein the region is responsible for causing at

least a portion of the symptoms. In one embodiment, the demyelinating disease or condition is multiple sclerosis. In a general embodiment, the composition (*i.e.*, cells) is autologous to the individual (*i.e.*, the cells are obtained from the same individual into whom they are then delivered).

5        In another aspect, the invention includes a method of purifying adult oligodendrocyte precursor cells from a suspension of cells derived from adult central nervous system (CNS) tissue. The method includes (i) contacting the suspension with a surface (*e.g.*, petri plastic surface, such as the inside of a plastic petri dish), derivatized to contain an antibody specifically immunoreactive with Thy-1. A portion of the cells in the  
10       suspension becomes immobilized on this surface, and a second portion of the cells remains non-adherent. The non-adherent portion is contacted with a second petri plastic surface derivatized to contain a moiety capable of selectively binding a marker preferentially-expressed on oligodendrocyte precursor cells. Examples of such moieties include the A2B5 antibody, the NG-2 monoclonal antibody and peanut agglutinin. Cells adhering to the  
15       second surface are substantially-purified adult oligodendrocyte precursor cells.

      In a related aspect, the invention includes a method of purifying adult oligodendrocyte precursor cells from a suspension of cells derived from adult central nervous system (CNS) tissue. The method includes (i) contacting the suspension with a surface (*e.g.*, petri plastic surface, such as the inside of a plastic petri dish), derivatized to  
20       contain an antibody specifically immunoreactive with proteolipid protein (PLP). In one embodiment, the surface also contains an antibody specifically immunoreactive with Thy-1. A portion of the cells in the suspension becomes immobilized on this surface, and a second portion of the cells remains non-adherent (*i.e.*, doesn't stick to the surface). The non-adherent portion is contacted with a second petri plastic surface derivatized to contain a  
25       moiety capable of selectively binding a marker preferentially-expressed on oligodendrocyte precursor cells. A preferred moiety for this application is an antibody directed against oligodendrocyte marker O4. Other suitable moieties include the A2B5 antibody, the NG-2 monoclonal antibody and peanut agglutinin. Cells adhering to the second surface are substantially-purified adult oligodendrocyte precursor cells.

30       In another aspect, the invention includes a method of determining relative numbers of adult oligodendrocyte precursors and perinatal oligodendrocyte precursors in a mixture of cells containing both cell types. Each cell in the mixture has a cytoplasmic portion and a nuclear portion. The method includes obtaining a sample of cells from the mixture, and assaying relative levels of expression of p53 in the nuclear and cytoplasmic portions of cells

in the sample. Perinatal oligodendrocyte precursors have higher levels of p53 expression in their cytoplasmic portion than in their nuclear portion, and adult oligodendrocyte precursors have lower levels of p53 expression in their cytoplasmic portion than in their nuclear portion.

- 5 In yet another aspect, the invention includes a method of identifying a compound capable of increasing the rate of proliferation of adult oligodendrocyte precursor cells. A sample of the cells is contacted with a test compound, the effect of the test compound on the rate of proliferation cells in the sample is measured, and the compound is identified as effective if its measured effect on the rate of proliferation is above a selected threshold
- 10 level. The threshold level may be selected, for example, to correspond to a selected number of standard deviations (s.d.) away from the mean rate of proliferation in the absence of test compounds.

- In another aspect, the invention includes a method of culturing adult oligodendrocyte precursor cells. The method includes incubating or growing the cells in a
- 15 medium which (i) is substantially free of feeder cells or medium conditioned by feeder cells, (ii) is substantially serum-free, and (iii) contains N-Acetyl-L-cysteine, PDGF and NT-3. The culturing results in maintenance of the adult oligodendrocyte precursor cells in an undifferentiated state.

- 20 These and other objects and features of the invention will become more fully apparent when the following detailed description is read in conjunction with the accompanying drawings.

#### **BRIEF DESCRIPTION OF THE FIGURES**

- 25 Figures 1A, 1B, 1C, 1D and 1E show a schematic diagram of a panning procedure for purifying adult oligodendrocyte precursor cells.

- Figures 2A and 2B show an oligodendrocyte precursor cell clone (Fig. 2A) and an oligodendrocyte clone (Fig. 2B) in cultures treated with NT-3 and PDGF. Note that the precursor cells have a bipolar morphology, while oligodendrocytes have multiple
- 30 interconnecting processes.

Figures 3A and 3B show the proliferative capacity of perinatal (Fig. 3A) and adult (Fig. 3B) oligodendrocyte precursor cells cultured for 12 days and 30 days, respectively, at clonal density in serum-free medium containing high insulin, PDGF, NT-3, and thyroid hormone (T3). Note that the perinatal precursor cells divided much more rapidly than the



adult precursor cells and that by 12 days most of the perinatal cells had given rise to clones of oligodendrocytes. Even by 30 days, however, none of the adult precursor cells had generated oligodendrocyte clones and the average cell had divided only about 3 times during the entire culture period.

- 5           Figures 4A and 4B show that perinatal precursor cells express cytoplasmic p53 staining (Fig. 4A), while adult precursor cells lack cytoplasmic staining but have nuclear staining (Fig. 4B).

#### DETAILED DESCRIPTION OF THE INVENTION

##### 10 I. Definitions

- The term "adult oligodendrocyte precursor cell", or "adult oligodendrocyte progenitor cell", refers to an oligodendrocyte precursor cell derived from a mammal whose CNS has matured to the point that essentially all axons destined to become myelinated have become myelinated (*e.g.*, a postnatal day 60 (P60) rat). Adult oligodendrocyte precursor
- 15 cells are similar to perinatal oligodendrocyte precursor cells but are distinguished from the perinatal cells in at least the following ways: adult oligodendrocyte precursor cells typically (i) contain more p53 antigen in their nucleus than their cytoplasm, (ii) have an endogenous cell cycle time of about 3-5 days (versus about 1 day for perinatal oligodendrocyte precursor cells), and (iii) divide about 5-10 times more slowly than perinatal
- 20 oligodendrocyte precursor cells.

The term "feeder cells" as applied to a culture of adult oligodendrocyte precursor cells is understood to mean other types of cells present in a culture to promote the survival, differentiation, growth or viability of the cultured adult oligodendrocyte precursor cells.

- The term "substantially pure", when used in connection with a culture of cells,
- 25 refers to a culture where over about 95% of the cells are of a single defined cell type. Similarly, the term "substantially purified", when used in reference to a particular population of cells, indicates that the population contains 95% or more of a single cell type.

- A suspension of cells "derived from" a tissue (*e.g.*, adult central nervous system (CNS) tissue, optic nerve, temporal lobe, etc.) refers to a cell suspension prepared from a
- 30 sample of that tissue (obtained using, *e.g.*, a biopsy or surgical procedure) by, for example, mincing the tissue and subjecting the minced tissue to enzymatic dissociation.

"Adult CNS tissue" refers to CNS tissue in which essentially all existing axons that are destined to become myelinated have been myelinated. In humans, such adult CNS tissue typically exists in individuals 3 years or more in age.

"Treating" a disease refers to administering a therapeutic substance effective to reduce the symptoms of the disease and/or lessen the severity of the disease.

## II. Oligodendrocytes and Oligodendrocyte Precursor Cells

- 5 Oligodendrocytes are relatively small cells that form myelin sheaths around axons of nerve cells of the mammalian central nervous system (CNS) by wrapping their processes concentrically around an axon in a tight spiral. A single oligodendrocyte typically envelopes several different axons (average of 15). Although there may be as many as 10,000 different types of nerve cells in the CNS, all myelinated axons in the CNS are  
10 myelinated by oligodendrocytes. The oligodendrocytes that myelinate the diverse collection of nerve cells in the CNS are morphologically indistinguishable, *i.e.*, they constitute a single "class" of cells. As such, oligodendrocytes isolated, *e.g.*, from the optic nerve, have the same properties as oligodendrocytes isolated, *e.g.*, from the spinal cord.

- Mature oligodendrocytes that form myelin sheaths are generated from  
15 oligodendrocyte precursor cells. At early stages in postnatal development, the majority of such oligodendrocyte precursor cells are "perinatal" cells. When cultured *in vitro*, these perinatal oligodendrocyte precursor cells can give rise either to type-2 astrocytes or oligodendrocytes, depending on the culture conditions. If the cells are cultured in serum-free medium, nearly all the cells differentiate into GC-positive oligodendrocytes. If, on the  
20 other hand, the cells are grown in the presence of serum, they differentiate into GFAP-positive type-2 astrocytes. *In vivo*, perinatal oligodendrocyte precursor cells appear to differentiate into oligodendrocytes, which then myelinate the developing axons. There is no compelling evidence that the type-2 astrocytes observed *in vitro* develop in the normal CNS *in vivo* (Skoff, 1990; Fulton, *et al.*, 1992). It has been reported, however, that significant  
25 numbers of oligodendrocyte precursor cells persist in the adult optic nerve, although they have largely ceased dividing (French-Constant and Raff, 1986; Wolswijk and Noble, 1989; Fulton *et al.*, 1992; Pringle *et al.*, 1992).

- Experiments performed in support of the present invention have shown that adult oligodendrocyte precursor cells *in vivo* divide at rates similar to those observed *in vitro*:  
30 About 3% of adult oligodendrocyte precursor cells were labeled one hour after intraperitoneal injection of the thymidine analog 5-bromo-2'-deoxyuridine (BRDU; available, *e.g.*, from Sigma Chemical Co., St. Louis, MO). A similar experiment on a perinatal animal resulted in about 20% of the perinatal oligodendrocyte precursor cells being labeled.

The adult oligodendrocyte precursor cells express a defining characteristic of stem cells in adult animals -- that is, they maintain the capacity to continually divide and differentiate. As such, these "adult" oligodendrocyte precursor cells may persist as a quiescent reservoir of stem cells in order to generate new precursor cells and

5 oligodendrocytes after axonal injury or demyelination; alternatively or additionally, the adult oligodendrocyte precursor cells may have other functions in addition to being precursor cells.

An exemplary system for the study of the relationship between oligodendrocytes, their precursor cells, and neurons is the optic nerve, where oligodendrocytes form myelin

10 sheaths around the axons of retinal ganglion cells (RGCs), which are CNS neurons that communicate signals from the retina of the eye to the brain. Most adult oligodendrocyte precursors arise in the rat optic nerve after postnatal day 14 (P14), at which point they constitute as many as 10% of the oligodendrocyte precursor cells. By P21, they constitute about 50%, and by P45, essentially 100% (Wolswijk *et al.*, 1990) of the oligodendrocyte

15 precursor cells. Adult precursor cells employed in the methods of the presently-described experiments were purified from the optic nerves of P60 rats. In humans, the time at which essentially 100% of oligodendrocyte precursors are adult oligodendrocyte precursors corresponds to the age at which essentially all existing axons that are destined to become myelinated have been myelinated, which is typically about 3 years of age.

20 Oligodendrocyte precursor cells in adult optic nerve cultures divide and differentiate more slowly *in vitro* than perinatal oligodendrocyte precursor cells cultured under the same conditions (Wolswijk *et al.*, 1990; Wren *et al.*, 1992). Adult oligodendrocyte precursor cells also migrate more slowly than perinatal precursor cells and have a different morphology and possibly antigenic phenotype as well (Wolswijk *et al.*, 1990).

25 Because all previous studies of adult precursor cells employed mixed optic nerve cultures that also contained astrocytes, oligodendrocytes, and other cell types, it has not been possible to determine whether the behavior of adult oligodendrocyte precursor cells differs from that of the perinatal cells because they are intrinsically different or because the extrinsic environment differs between adult and perinatal animals.

30 Timelapse studies of oligodendrocyte precursor cells have suggested that at least some adult oligodendrocyte precursor cells may be derived directly from a subpopulation of perinatal precursors (Wren *et al.*, 1992) and that perinatal and adult precursor cells could co-exist in the same culture environment, suggesting that the two cell types were intrinsically different.

In apparent opposition to this view, however, it was recently reported that adult oligodendrocyte precursor cells cultured in the presence of both PDGF and bFGF behave identically to perinatal precursor cells (Wolswijk and Noble, 1992), suggesting that their behavior is not intrinsically specified as either perinatal and adult phenotypes, but rather that these are two intraconvertible phenotypes that depend on the local environment.

Results of experiments performed in support of the present invention indicate that adult and perinatal oligodendrocyte precursor cells are intrinsically different, and that this difference is not simply due to the environment in which the cells are placed. The results also suggest, however, that perinatal cells may be induced to take on the characteristics of the adult cells, as the rate of cell division of purified perinatal oligodendrocyte precursor cells cultured for several weeks slows down with age, *i.e.*, cultured perinatal oligodendrocyte precursor cells divide progressively more slowly with increasing age.

The present invention provides, in one aspect, a method for the isolation and culturing of adult oligodendrocyte precursor cells. The isolation of adult oligodendrocyte precursor cells facilitates screening for compounds effective to increase the proliferation of the cells. Further, such cells may be used to remyelinate axons following neuronal injury or in treatment of demyelinating diseases. The present invention also enables the growing and expansion of pure or substantially pure cultures of adult oligodendrocyte precursor cells in culture in order to transplant the cells into sites of neuronal injury for therapeutic applications.

### III. Purification and Culture of Adult Oligodendrocyte Precursor Cells

#### A. Panning Purification

According to the methods of the present invention, adult oligodendrocyte precursor cells can be purified from any suitable CNS tissue source using one of the adaptations of immunopanning procedures (Barres, *et al.*, 1992, 1993b, 1994b, Wysocki and Sato, 1978; Mage, *et al.*, 1977) described below. Exemplary sources of tissue include, in the case of experimental animals, as postnatal optic nerve; in the case of humans, brain biopsies or surgically-removed samples of, *e.g.*, right temporal lobe.

The brain tissue is preferably isolated from an individual of a developmental age at which most of oligodendrocyte precursor cells are adult oligodendrocyte precursor cells. For example, in the case of rat, the tissue is isolated from animals that are preferably at least about 45 days old, as it has been demonstrated that at this stage, virtually all of the

oligodendrocyte precursor cells are adult oligodendrocyte precursor cells (Wolswijk, *et al.*, 1990).

In the case of humans, biopsies of CNS tissue may be obtained from patients over the age of about 3 years who are, for example, in need of autologous oligodendrocyte cell-replacement therapy.

Cells are isolated from the tissue to form a tissue suspension (*e.g.*, as described in Materials and Methods, herein).

In one embodiment, described in Example 1, below, adult oligodendrocyte precursor cells are isolated from rat optic nerve. The cell suspension is then depleted of Thy1.1-positive cells, such as macrophages, astrocytes, meningeal cells and microglia. Thy1.1, a member of the Ig superfamily, is one of the best-characterized antigens in biology. It was originally identified on thymocytes (Williams and Gagnon, 1982), and was used to differentiate T-cells from B-cells. In the brain, Thy-1 is a major glycoprotein found on the surface of many neurons, mature oligodendrocytes, astrocytes, microglia, macrophages and the like.

A number of antibodies directed against Thy1.1 have been generated, and some are available from the ATTC (*e.g.*, hybridoma cell line T11D7e2; Accession number TIB 103, which contains IgM antibodies against Thy1.1) or through commercial sources (*e.g.*, monoclonal antibody OX-7 is available from Serotec, Oxford, United Kingdom; another anti-Thy1.1 antibody is available from Boehringer- Mannheim (Indianapolis, IN) as Cat.# 1199200). Any suitable anti-Thy1.1 antibodies may be employed to remove Thy1.1-positive cells from the suspension. For instance, in experiments described below, OX-7 monoclonal antibody was used for this purpose. The mouse OX-7 monoclonal was coated onto a dish that had been derivatized with a goat anti-mouse antibody, and the dish was used in a standard panning protocol as described in Example 1.

The depletion of Thy1.1-positive cells is typically accomplished in a single panning step, but may include two or more rounds of panning if a large number of Thy1.1-positive cells are present in the suspension. The decision as to whether or not to include additional anti-Thy1.1 steps may be based in part on the relative number of Thy1.1-positive cells that are present in the non-adherent cell suspension following the initial anti-Thy1.1 panning step. The anti-Thy1.1 panning steps together typically remove over about 99% (preferably about 99.9%) of the Thy1.1-positive cells that were present in the original suspension.

After depletion of Thy1.1-positive cells, the suspension is transferred to a plate derivatized with an antibody preferentially-expressed on oligodendrocyte precursor cells.

An exemplary antibody useful for this purpose is the monoclonal antibody secreted by the A2B5 hybridoma cell line, available from the American Type Culture Collection (ATCC; Rockville, MD) under accession number CRL 1520. The A2B5 antigen is expressed on both perinatal and adult oligodendrocyte precursor cells, but is not expressed on other cells found in the optic nerve, such as mature oligodendrocytes, neurons and microglia. Occasional pial fibroblasts that encapsulate the nerve are Thy-1 positive and A2B5 positive. These cells are eliminated using the first (anti-Thy-1) panning plate. Because the source material for this purification contains virtually no perinatal oligodendrocyte precursor cells (*i.e.*, it is obtained from adults), only adult oligodendrocyte precursor cells stick to the second panning plate (the "positive-selection" plate).

The purified adult oligodendrocyte precursor cells are detached from the "positive selection" dish (*e.g.*, A2B5 dish) using trypsin. The yield obtained with the procedure described in Example 1 was about 2,000 cells per adult rat (1,000 per nerve). This yield is consistent with the total yield of cells in the adult suspension: there are about 600,000 cells in an adult rat optic nerve (Barres, *et al.*, 1992) of which approximately 15% can be isolated enzymatically (90,000). The adult optic nerve contains about 8,000 oligodendrocyte precursor cells (Fulton, *et al.*, 1992). Accordingly, the number of adult oligodendrocyte precursor cells isolated using the methods of the present invention is proportional to the total cell yield, indicating that these methods are effective to isolate a representative sample, as opposed to a special subset, of adult oligodendrocyte precursor cells.

Control experiments performed in support of the present invention demonstrated that few if any oligodendrocyte precursor cells were non-adherent to the final dish, consistent with their known A2B5-positive phenotype.

In another embodiment, described in Example 5, the adult oligodendrocyte precursor cells are purified as described above, except that an antibody directed against PLP is used instead of or in addition to an anti-Thy1.1 antibody in the first panning plate. The second panning plate contains, as above, an antibody expressed on adult oligodendrocyte precursor cells. However, since mature oligodendrocytes are removed in the first step by binding to the anti-PLP antibody, the second (positive selection) step can employ antibodies expressed on oligodendrocytes as well as on oligodendrocyte precursor cells. An exemplary antibody suitable for use with the second panning plate is one directed against oligodendrocyte antigen O4. Other antibodies may be used as well, including those used in the embodiment described in Example 1.

B. Affinity Reagents Useful in Purification of Adult Oligodendrocyte Precursor Cells

The final step in the purification method detailed above employs antibodies directed against cell surface markers expressed on oligodendrocyte precursor cells, but preferably not on the other cell types typically found in association with such precursor cells (such as mature oligodendrocytes, astrocytes, neurons, microglia and the like). The method detailed in Example 1, below employs an exemplary antibody -- A2B5, which is immunoreactive with a ganglioside specifically expressed on oligodendrocyte precursor cells (Eisenbarth, *et al.*, 1979). Although this antigen is expressed in the embryonic CNS on radial glia, other precursor cells and some neurons, it is not expressed on these cells in the adult CNS.

Another method of achieving the final panning step is by using the lectin peanut agglutinin. Experiments performed in support of the present invention have shown that peanut agglutinin specifically binds to both perinatal and adult oligodendrocyte precursor cells, and that it may be used in the panning procedure described above to isolate a purified population of oligodendrocyte precursor cells. Peanut agglutinin is commercially available (*e.g.*, from Vector Labs, Burlingame, CA, as Cat. # L-1070).

Alternatively, one of skill in the art may elect to generate antibodies against antigens specifically expressed on oligodendrocyte precursor cells, and use such antibodies in the second (positive selection) panning plate. Material isolated or derived from either perinatal or adult precursor cells may be used to immunize an animal for the generation of such antibodies. This approach has been used to generate the NG-2 monoclonal antibody (Stallcup and Bersley, 1987), directed against the NG-2 proteoglycan, which is specific for oligodendrocyte precursor cells.

An antibody specifically immunoreactive with the oligodendrocyte antigen O4 may also be used in the second panning plate. Reports describing the generation of such antibodies have been published (*e.g.*, Sommer and Schachner, 1981, 1982; Gogate, *et al.*, 1994). Additionally, anti-O4 antibodies specifically immunoreactive with human O4 are commercially available (*e.g.*, from Boehringer-Mannheim, Indianapolis, IN).

The material used for immunization may include, for example, cell membrane preparations or fractions, proteins (*e.g.*, membrane proteins) purified from the cells, glycoproteins or gangliosides or recombinantly-produced material from the precursor cells (*i.e.*, a recombinant protein expressed using a vector containing DNA sequences encoding an antigen expressed on the precursor cells). Recombinant methods of producing antigenic material may employ fusion proteins to facilitate purification of the antigenic polypeptide.

Hybrid, or fused, proteins may be generated using a variety of coding sequence derived from other proteins, such as glutathione-S-transferase or  $\beta$ -galactosidase.

Antibodies may be generated by immunizing a suitable animal according to methods known in the art (Harlow, *et al.*). Antigenic materials may be used directly for the generation of antibodies, or they may be coupled to appropriate carrier molecules. Many such carriers are known in the art and are commercially available (*e.g.*, Pierce, Rockford IL). A recombinant antigen may also be produced as a fusion protein in tandem with a polypeptide carrier molecule.

To prepare antibodies, a host animal, such as a rabbit, is typically immunized with the antigenic material. The host serum or plasma is collected following an appropriate time interval, and the serum is tested for antibodies specific against the antigen.

The gamma globulin fraction or the IgG antibodies of immunized animals can be obtained, for example, by use of saturated ammonium sulfate precipitation or DEAE Sephadex chromatography, affinity chromatography, or other techniques known to those skilled in the art for producing polyclonal antibodies.

Alternatively, purified antigen or fused antigen protein may be used for producing monoclonal antibodies. In this case, the spleen or lymphocytes from an immunized animal are removed and immortalized or used to prepare hybridomas by methods known to those skilled in the art (*e.g.*, Harlow, *et al.*). Antibodies secreted by the immortalized cells are screened (*e.g.*, using enzyme linked immunosorbent assay (ELISA) or a Western blot) to determine the clones that secrete antibodies of the desired specificity (*e.g.*, Ausubel, *et al.*). The screens may also include staining of samples (*e.g.*, tissue culture cells or optic nerve tissue samples) to identify antibodies directed against cell-specific (*e.g.*, precursor cell-specific) antigens.

#### C. Purity of Adult Oligodendrocyte Precursor Cells

Experiments performed in support of the present invention demonstrate that adult oligodendrocyte precursor cells purified by the methods of the present invention are typically greater than 99% pure.

#### D. Differences in Purification of Adult and Perinatal Oligodendrocyte Precursor Cells

Methods for purifying perinatal oligodendrocyte precursor cells have been previously described (Barres, *et al.*, 1992; Barres, 1993a). These methods also employed a



panning protocol like that used in the present methods. However, the strategy used in the methods of the present invention is considerably different.

In the previously-described methods (Barres, *et al.*, 1992; Barres, 1993a), the purification employed anti-RAN-2 antibodies and anti-GC (anti-galactocerebroside glycolipid) antibodies to separate out contaminating cells. RAN-2 is an unknown protein that is specifically expressed on optic nerve type-1 astrocytes (Bartlett, *et al.*, 1981), while GC is a glycolipid expressed specifically on oligodendrocytes. The anti-RAN-2 step depleted type-1 astrocytes, meningeal cells, microglia and macrophages, while the anti-GC step depleted mature oligodendrocytes.

Experiments performed in support of the present invention demonstrated, however, that adult oligodendrocyte precursor cells adhered both to the RAN-2 and GC dishes. This observation ruled out the use of these previously-described methods for the purification and isolation of adult oligodendrocyte precursor cells.

#### 15 E. Culture of Adult Oligodendrocyte Precursor Cells

Utility of adult oligodendrocyte precursor cells isolated as described herein may be enhanced by maintaining the cells under conditions which support survival and expansion of the cells in the absence of substantial differentiation.

The fate of adult oligodendrocyte precursor cells isolated as described herein depends on the culture conditions. For example, more than 95% of the cells differentiate into GC-positive oligodendrocytes when cultured in serum-free medium lacking mitogens but containing survival factors such as insulin and CNTF. However, more than 95% of the cells differentiate into GFAP-positive type-2 astrocytes when cultured in medium containing 10% FCS.

Additional experiments showed that when the adult oligodendrocyte precursor cells purified by the methods of the present invention were grown in medium containing PDGF, NT-3 and insulin, more than 95% of the cells divided. This result indicates that the purified cells are not postmitotic oligodendrocytes. Further, nearly all the dividing cells were A2B5-positive and GC-negative, as expected for precursor cells.

It was also found that the inclusion of N-Acetyl-L-cysteine to a serum-free medium containing mitogens, such as PDGF and NT-3, allowed the cells to continue dividing, as opposed to differentiating.

These results further indicate that the cells can be maintained for extended periods in culture as undifferentiated precursor cells for subsequent use with, *e.g.*, the methods

described herein. In particular, according to the data herein, an exemplary medium for maintaining the cells in such an undifferentiated state is the modified Bottenstein and Sato serum-free medium minus T3 and T4 thyroid hormone, and including at least one survival factor (e.g., insulin), mitogens such as NT-3 and PDGF, and N-acetyl-L-cysteine.

5

IV. Characterization of Adult Oligodendrocyte Precursor Cells and Comparisons with Perinatal Oligodendrocyte Precursor Cells

A. Antibody Staining

Perinatal and adult oligodendrocyte precursor cells share a number of common cell-  
10 surface markers; which may be used to identify as well as purify oligodendrocyte precursor cells. The markers that have been identified thus far as being expressed on both cell types include A2B5, O4, NG-2 and peanut agglutinin-binding carbohydrates.

Additional experiments performed in support of the present invention have resulted  
15 in the identification of an antigenic marker (p53) that may be used to differentiate adult from perinatal precursors. p53 is a transcription factor that acts in the nucleus to suppress or slow the cell cycle, and that is sequestered from acting by binding to cytoplasmic proteins (Donehower and Bradley, 1993; Berns, 1994).

Experiments performed in support of the present invention demonstrate that a  
commercially-available monoclonal antibody (pAb240, Cat.# OP29, Oncogene Science,  
20 Uniondale, NY), which binds to both human and rat p53, differentially immunostains perinatal and adult oligodendrocyte precursor cells. Perinatal precursor cells exhibit bright cytoplasmic labeling, whereas adult precursor cells entirely lack cytoplasmic staining but have nuclear labeling. The labeling pattern is not dependent on the stage of the cell cycle, because all cells shared the same labeling pattern regardless of their cell cycle stage.

25

B. Differentiation of Perinatal Oligodendrocyte Precursor Cells in Cultures Plated at Clonal Density

The proliferation and differentiation behavior of purified perinatal and adult  
oligodendrocyte precursor cells was compared. Previous studies have shown that the  
30 proliferation and differentiation of perinatal oligodendrocyte precursor cells can be studied *in vitro* at clonal density under completely defined serum-free conditions in which oligodendrocyte precursor cells replicate many aspects of their normal *in vivo* behavior (Barres and Raff, 1994).

The studies were performed as follows: Oligodendrocyte precursor cells were  
35 purified from postnatal rat optic nerve cell suspensions to greater than 99.95% purity by

sequential immunopanning (Barres, *et al.*, 1992). The purified cells were cultured at clonal density in a serum-free medium that contained transferrin, progesterone, putrescine, selenium, thyroxine, triiodothyronine, albumin, and a high concentration (5 µg/ml) of insulin (modified from Bottenstein and Sato, 1979; see Materials and methods, below). In this medium, all of the cells prematurely differentiated into oligodendrocytes during the first 4 days of culture, as expected in the absence of mitogens.

Further, when either NT-3 or PDGF was added at plateau concentrations (Barres, *et al.*, 1994a) to the culture medium, all of the cells also differentiated into oligodendrocytes after 4 days of culture, although some of the cells under these conditions divided once prior to differentiating. The percentage of cells that divided once in either NT-3 or PDGF was similar to the percentage that could be induced to synthesize DNA by NT-3 or PDGF (Barres, *et al.*, 1993b).

Clonal expansion did occur, however, when the cells were cultured in NT-3 and PDGF together: most of the cells now divided more than once over 4 days of culture and the cells in more than half of the clones were still precursors rather than oligodendrocytes. In medium containing NT-3 and PDGF, some clones were still expanding after 16 days. In the presence of both NT-3 and PDGF, oligodendrocyte differentiation occurred synchronously within a clone, indicating that the intrinsic clock that limits the maximal number of divisions operates under these conditions.

The results described above demonstrate that PDGF and NT-3 collaborate to promote oligodendrocyte survival, proliferation and differentiation *in vitro*; under these culture conditions, the cells closely replicate their *in vivo* behavior (Barres, *et al.*, 1994b).

In addition, it was found that delivery of a neutralizing antibody to NT-3 into the developing optic nerve reduces in half the rate of oligodendrocyte precursor cell proliferation as well as the number of oligodendrocytes that develop (Barres, *et al.*, 1993b, 1994b). Consistent with this possibility, experiments performed in support of the present invention have shown that there are high levels of NT-3 protein in P14 optic nerves (about 10 ng/g total protein), which is the peak time of oligodendrocyte precursor cell proliferation during development.

Additional experiments performed to measure the amount of PDGF-AA by ELISA in P14 optic nerves revealed high levels of PDGF protein in P14 optic nerves (about 50 ng/g). Together these findings demonstrate that PDGF and NT-3 normally act together to promote the development of oligodendrocytes.

Other experiments performed in support of the present invention have shown that thyroid hormone signals the clock mechanism that induces oligodendrocyte precursor cells to stop dividing and differentiate. When thyroid hormone (T4 and T3) was eliminated from the serum-free medium, oligodendrocyte precursor cells were still able to divide in response to PDGF and NT-3, but they did not differentiate into oligodendrocytes (Barres, *et al.*, 1994b). Further experiments showed that this effect was not attributable to an effect on survival or differentiation. Rather, the results indicate that thyroid hormone is required for oligodendrocyte precursor cells to stop dividing in response to mitogens. As the appearance of oligodendrocytes in the developing optic nerve normally coincides with the onset of thyroid function and can be altered by altering thyroid hormone levels *in vivo*, these findings indicate that the clock mechanism that limits the maximum number of perinatal oligodendrocyte precursor cell divisions is controlled by thyroid hormone.

In view of the results presented above, it can be appreciated that under appropriate culture conditions, adult oligodendrocyte precursor cells can be maintained in culture for extended periods (at least one month) in a proliferating, non-differentiated state suitable for expansion of the cells *in vitro*. These "appropriate" culture conditions include the use of a serum-free culture medium containing survival factors (*e.g.*, insulin), the mitogens NT-3 and PDGF, and N-acetyl-L-cysteine. Such a medium also preferably does not contain thyroid hormone.

C. Differentiation of Adult Oligodendrocyte Precursor Cells in Cultures Plated at Clonal Density

To compare the properties of the adult and perinatal oligodendrocyte precursor cells, adult (P60) and P8 perinatal precursor cells were purified simultaneously. The characteristics of the cells were compared when the cells were cultured at clonal density in the same serum-free culture medium containing their mitogens PDGF, NT-3, insulin, as well as thyroid hormone to activate the clock mechanism. After 12 and 30 days, the clones were counted and the identity of each cell scored as an oligodendrocyte or oligodendrocyte precursor cell, depending on morphology and expression of GC.

As shown in Fig. 3B, the average cell cycle time of the adult precursor cells is about 10 days (in the presence of T3; in the absence of T3 the adult precursor cell cycle time is about 5 days), which is about 10 times longer than that of the perinatal precursor cells. This result was observed in 3/3 experiments. Moreover, whereas clones of oligodendrocytes appeared and accumulated as expected in the perinatal cultures, so that after prolonged culture times the majority of the clones were oligodendrocyte clones, this

was not the case with the adult cells, even after prolonged culture periods to allow them to make an equivalent number of divisions. Very few if any clones of oligodendrocytes were generated by the adult oligodendrocyte precursor cells (Fig. 3B).

- 5 *In vitro*, over 95% of the adult oligodendrocyte precursor cells grown in serum-free medium differentiated into oligodendrocytes while over 95% of the adult oligodendrocyte precursor cells grown in medium containing 10% fetal calf serum differentiated into type-2 astrocytes. Further, over 95% of the adult oligodendrocyte precursor cells cultured *in vitro* in serum-free medium containing mitogens (e.g., PDGF and NT-3) and at least one survival factor (e.g., insulin) divided.

10

#### V. Uses of Purified Adult Oligodendrocyte Precursor Cells

##### A. Demyelinating Diseases and Conditions

- Demyelinating diseases are a group of neurologic disorders significant both because of the disability that they cause and the frequency with which they occur. Demyelinating diseases are characterized by patchy or focal destruction of myelin sheaths in the CNS accompanied by an inflammatory response. The most common demyelinating disease is multiple sclerosis. Other examples include acute disseminated encephalomyelitis and acute hemorrhagic leukoencephalitis.

- 20 Multiple sclerosis is generally manifested by recurrent attacks of focal or multifocal neurologic dysfunction. The symptoms are determined by the location of foci, or plaques, of demyelination within the CNS. Classic features include impaired vision, nystagmus, dysarthria, decreased perception of vibration and position sense, ataxia and intention tremor, weakness or paralysis of one or more limbs, spasticity, and bladder problems. The precise locations of these plaques can be determined using, e.g., magnetic resonance imaging (MRI).

25

Demyelination of CNS axons can also occur during acute or traumatic brain injury, such as during spinal cord injury. According to the present invention, any condition which results in CNS axons losing their myelin sheaths may be amenable to therapeutic methods described herein.

30

##### B. Remyelination of Damaged Axons

Oligodendrocyte precursor cells are the stem cells responsible for myelination. In the developing animal, perinatal oligodendrocyte precursor cells generate large numbers of oligodendrocytes, which myelinate the newly-developing axons. Adult oligodendrocyte

precursor cells may also be responsible for the limited remyelination that occurs following certain types of neuronal injury. In many cases, however, the condition of a patient suffering from a demyelinating disease or condition could be greatly improved if the rate and/or degree of remyelination could be accelerated.

- 5 According to the present invention, adult oligodendrocyte precursor cells may be isolated from patients with a neuronal injury, expanded in culture, and transplanted back into the patient to facilitate remyelination. Such *in vitro* expansion/transplantation methods are routinely used in several areas of medicine, including hematopoietic cell replacement (Eaves, *et al.*, 1993; Koller, *et al.*, 1993; Rummel and Van Zant, 1994; Silva, *et al.*,  
10 1995), skin grafts in burn patients (*e.g.*, Rheinwald and Green, 1975; Ronfard, *et al.*, 1991; Teepe, *et al.*, 1990), and are contemplated in other areas, *e.g.* bone and cartilage reconstruction (*e.g.*, Brent, 1992; Nakahara, *et al.*, 1991).

- The cells are preferably isolated from a region of the brain whose removal results in little or no disruption of the individual's mental functioning and that is preferably unaffected  
15 by the demyelinating disease or condition. For example, the cells may be isolated from a region in the individual's right temporal lobe.

- Selected portions of the temporal lobe are routinely removed in patients suffering from focal temporal lobe epilepsy that is refractory to medical therapy (Son, *et al.*, 1994; Shih, *et al.*, 1994; Benbadis, 1995). Such surgery is safe, well-known and accepted and,  
20 when candidates are selected appropriately, yields excellent results with few if any adverse effects. Since the methods described herein typically require the removal of significantly smaller portions of brain tissue, any potential adverse side effects are further minimized.

- Alternatively, autologous human brain tissue may be isolated from a suitable region of the brain (*e.g.*, the right temporal lobe) using a biopsy procedure, such as a computed  
25 tomography (CT)-guided needle biopsy or stereotactic biopsy (Wen, *et al.*, 1993).

The tissue is prepared, and human adult oligodendrocyte precursor cells are purified as described, *e.g.*, in Examples 1 or 5.

- Adult oligodendrocyte precursor cells purified as described above can be used immediately, or can be maintained in culture under conditions which support cell division  
30 and inhibit differentiation as described herein.

Adult oligodendrocyte precursor cells purified as described above, and optionally expanded in culture, can be transplanted at the site of injury using known methods, *e.g.*, as described for transplantation of fetal cells into brains of Parkinson's patients (see, *e.g.*, Molina, *et al.*, 1994; Kupsch and Oertel, 1994). A selected number of cells (*e.g.*, 5,000-

50,000) are suspended in a small volume of a buffer (e.g., sterile, isotonic PBS) compatible with the ionic environment of the area of the brain into which the cells are being delivered, and the suspension is delivered (e.g., via injection), to the injured area. The injection does not necessarily need to be precisely at the site of the lesion, since the oligodendrocyte precursor cells are known to migrate to demyelinated axons. The number of cells injected depends on several factors, such as the availability of source tissue, whether or not the cells were expanded, the size of the injured area and the like.

For example, to treat MS, adult oligodendrocyte precursor cells purified from a portion of the brain unaffected by demyelinated plaques are optionally expanded in culture and transplanted, implanted or injected into regions of symptomatic plaques -- that is, into plaques (localized using, e.g., MRI) that are in areas which correspond to the clinically-diagnosed neurological deficit.

### C. Population Studies of Purified Cultures vs Clonal Analysis of Single Cells

The methods described herein offer a number of advantages over a traditional clonal analysis technique of micromanipulation of single cells from mixed cell type suspensions, as has been described for perinatal oligodendrocyte precursor cells by Temple and Raff, 1985, 1986. The advantages include the following. First, a much larger number of cell clones can be examined. Thus, in a single experiment the behavior of thousands of oligodendrocyte precursor cells cultured under various conditions can be studied (compared to perhaps 30 oligodendrocyte precursor cells that can be micromanipulated into wells in a single experiment).

Second, the presently-described methods enable the determination of the average behavior of single oligodendrocyte precursor cells, avoiding selection or sampling errors. For instance, selection errors may have been introduced when these cells were micromanipulated if certain types of cell morphologies were preferentially selected.

Third, because relatively large populations of cells of a single type can be purified relatively easily, the cells can be employed in screening applications (such as is described below) in which it is desirable to detect changes (due to, for example, the application of a proliferation-inducing compound) affecting the entire population of cells in a consistent way. Such screening applications would be difficult, if not impossible, to carry out using a cells purified one-at-a-time using the traditional clonal analysis technique.

D. Screen for Compounds Capable of Accelerating Proliferation

An exemplary utility of the purified adult oligodendrocyte precursor cells described herein involves their use in a screen for compounds effective to treat neurological injury and demyelinating diseases. Compounds identified by such screens preferably increase the  
5 rate of remyelination at the injured/diseased sites when administered to subjects in need of treatment.

The screen may be carried out as follows: A sample of the cells is contacted with a test compound, the effect of the test compound on the rate of proliferation cells in the sample is measured, and the compound is identified as effective if its measured effect on the  
10 rate of proliferation is above a selected threshold level. The threshold level may be selected, for example, to correspond to a selected number of standard deviations (s.d.) away from the mean rate of proliferation in the absence of test compounds. The threshold level is set by the practitioner of the invention to a level corresponding to the desired potency of the test compound. For example, if the above-described screen is employed as a pre-screen  
15 to identify compounds for further detailed analyses, the threshold level may be set such that it corresponds to a relatively small change in rate of proliferation (e.g., 2-3 s.d.).

Alternatively, if the screen is being used as a final step in the identification of compounds having a potent effect on proliferation, the threshold level may be set, for example, to 4-6 s.d. relative to the mean rate in absence of compound, or to a 2-fold or  
20 greater difference relative to the mean rate in absence of compound.

Several different *in vitro* characteristics can be employed as the criteria for such a screen. For example, the cells can be screened for compounds effective to increase the rate of proliferation or cell division of the adult precursor cells. The rate of proliferation may be assayed as described above.

Compounds identified as effective in such a screen may be used to increase the rate of cell division in cultures grown for cell transplantation therapy. Alternatively, the compounds may be administered to individuals suffering from a neuronal injury or neurodegenerative disease which could benefit from remyelination therapy. Examples of such diseases include multiple sclerosis and other demyelinating diseases, as well as cerebral  
25 palsy and glaucoma. Further, since trauma to the CNS typically results in demyelination, such compounds may be effective at treating traumatic CNS injury, such as spinal cord injury.  
30

The cells may also be screened for the ability to revert to cells having the characteristics of perinatal oligodendrocyte precursor cells. According to the present



disclosure, and exemplary indicator for such a reversion is immunostaining with anti-p53 antibody, which can differentiate between perinatal and adult oligodendrocytes. Compounds effective to cause such a reversion may be employed in applications such as those described above.

- 5           A variety of different compounds may be screened using the above approaches. They include peptides, macromolecules, growth factors, small molecules, chemical and/or biological mixtures, and fungal, bacterial, or algal extracts and the like. Such compounds, or molecules, may be either biological, synthetic organic, or even inorganic compounds, and may be obtained from a number of sources, including pharmaceutical companies and  
10   specialty suppliers of libraries (*e.g.*, combinatorial libraries) of compounds.

The following examples illustrate but in no way are intended to limit the present invention.

15

#### MATERIALS AND METHODS

Unless otherwise indicated, chemicals were purchased from Sigma (St. Louis, MO).

##### A. Buffers

##### **Phosphate-buffered saline (PBS)**

- 20           10x stock solution, 1 liter:  
            80 g NaCl  
            2 g KCl  
            11.5 g  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$   
            2 g  $\text{KH}_2\text{PO}_4$   
25           Working solution, pH 7.3:  
            137 mM NaCl  
            2.7 mM KCl  
            4.3 mM  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$   
            1.4 mM  $\text{KH}_2\text{PO}_4$

30

##### B. Animal Procedures

- Cells for *in vitro* experiments were obtained by sacrificing the animals (typically rats) and obtaining the appropriate tissue by dissection. The animals were sacrificed either by fluothane inhalation anesthesia (adult rats) or by fluothane inhalation anesthesia followed  
35   by decapitation with a sharp pair of scissors (rat pups).

### C. Growth Factors

- Recombinant human insulin-like growth factor 1 (IGF-1) and insulin-like growth factor 2 (IGF-2) were obtained from Peprotech (Rocky Hill, NJ). Insulin was obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant mouse neurotrophin-3 (NT-3) was obtained from Yves Barde (Gotz, *et al.*, 1992, Max Plank Institute for Psychiatry, Martinsried, Federal Republic of Germany). Recombinant rat ciliary neurotrophic factor (CNTF) was obtained from Michael Sendtner and Hans Thoenen (Stockli, *et al.*, 1989, Max Plank Institute for Psychiatry), and platelet-derived growth factor (PDGF) was obtained from Peprotech, Rocky Hill, NJ.
- The growth factors may also be obtained from other commercial sources, *e.g.*, Genzyme Diagnostics (Cambridge, MA) and R&D Systems (Minneapolis, MN).

### D. Dissection and Dissociation of Optic Nerve.

- Optic nerve was obtained from postnatal day 60 (P60) Sprague Dawley (S/D) rats (Simonsen Labs, Gilroy, CA). The animal was decapitated, the optic nerves and optic chiasm were dissected with micro-dissecting forceps and small scissors, collected in 35 mm petri dishes containing 2 ml of Minimal Essential Medium (MEM) supplemented with 10 mM Hepes (MEM/Hepes), and minced using small scissors.
- The optic nerve was then dissociated enzymatically to make a suspension of single cells, essentially as described by Huettner and Baughman (1986).

- A papain solution was prepared, immediately prior to the start of the dissection, by adding 300 units of papain (Worthington Biochemical, Freehold, NJ) to 10 ml of Earle's Balanced Salt Solution (EBSS; Gibco/BRL Life Technologies, Gaithersburg, MD) in a 15 ml blue-top conical centrifuge tube, and placing the mixture in a 37°C water bath to dissolve the papain. One hundred microliters of a 4 mg/ml DNase (0.004%, Worthington Biochemical Corp., Freehold, NJ) solution were added to the MEM/papain mixture after the papain had dissolved. About 10 minutes before use, the solution was mixed with 2.4 mg of L-cysteine, adjusted to a pH of about 7.4 with 1M NaOH, and passed through a 0.22 micron filter into sterilized scintillation vials.

- Upon completion of the dissection, the MEM bathing the tissue was removed with a sterile pasteur pipette and replaced with 2 ml of the papain solution. The tissue was then decanted to a scintillation vial containing fresh papain solution, and the vial was placed in a 37°C water bath for 30 minutes with gently swirling approximately every 10 minutes.

The tissue and papain solution in the scintillation vial were then decanted to a 15 ml blue-top centrifuge tube. After the tissue settled to the bottom, the old papain solution was removed with a sterile pipet and the tissue was gently rinsed with 3 ml of ovomucoid inhibitor solution, which contained ovomucoid (15 mg; Boehringer-Mannheim) and BSA (10 mg; Sigma catalog no. A-7638) dissolved in MEM (GIBCO/BRL). The solution was adjusted to pH 7.4 and sterilized with a 0.22  $\mu$ m filter). The pieces of tissue were allowed to settle, and the rinse solution removed.

The tissue was then triturated sequentially with #21 and then #23 gauge needles. The following steps were repeated 6-10 times (until the tissue was completely broken up):

- (i) one ml of ovomucoid solution was added and the tissue was gently pulled up into the needle and expelled, (ii) the dissociate was allowed to settle by gravity for about 30 seconds, and (iii) the supernatant was collected. The final cell suspension, comprised of the supernatants from the 6-10 trituration cycles, contained about 50,000 cells per P8 optic nerve.

The cell suspension was then spun at 800 Xg for 10 minutes in a 15 ml blue-top centrifuge tube to separate the cells from the ovomucoid solution. The supernatant was discarded and cells resuspended in 1 ml of MEM. The cell suspension was then gently layered onto 6 ml of an MEM solution containing 60 mg of ovomucoid and 60 mg of BSA (pH adjusted to pH 7.4) and spun again at 800 Xg for 10 minutes in a 15 ml blue-top centrifuge tube. The supernatant was discarded and the cells were resuspended in 12 ml of Eagle's Minimum Essential Medium (MEM) solution containing BSA (0.1%). During this procedure the cells were never exposed to glutamate, aspartate or glutamine, or allowed to be cooled lower than room temperature.

#### 25 E. Tissue Culture

Purified cells were typically cultured in 96-well plates (Falcon) that had been coated with merosin (2  $\mu$ g/ml; Telios Pharmaceuticals Inc., San Diego, CA, available from Gibco/BRL) in 100  $\mu$ l of modified Bottenstein-Sato (MBS) serum-free medium. The percentage of surviving cells was assessed after 3, 7, and 14 days by the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (see below). All values were normalized to the percentage of surviving cells at 3 hours after plating, which represented the percentage of cells that survived the purification procedure. This initial viability was typically about 85%.

The MBS medium was similar to Bottenstein-Sato (B-S) medium (Bottenstein and Sato, 1979), but used "NEUROBASAL" (Gibco/BRL), instead of Dulbecco's Modified Eagle's Medium (DMEM), as the base. "NEUROBASAL" is a recently-described basal medium that has been optimized for neuronal cell culture (Brewer, *et al.*, 1993).

- 5 The serum-free components added to the "NEUROBASAL" base to make MBS medium were bovine serum albumin (BSA), selenium, putrescine, thyroxine, tri-iodothyronine, transferrin, progesterone, pyruvate, glutamine and N-Acetyl-L-cysteine. The N-Acetyl-L-cysteine was found to potentiate the effects of all oligodendrocyte precursor cell mitogens, such as PDGF. It was included in the medium to allow the cells to continue
- 10 dividing, as opposed to differentiating. Various trophic factors and other additives were added as indicated in individual experiments. The MBS medium was prepared with a highly purified, crystalline grade of BSA (Sigma, A4161), in order to avoid contaminating survival factors.

- The component concentrations of the MBS medium used in the present experiments
- 15 are provided in Table 1, below.

**Table 1**

**MBS COMPONENTS**

20

Component	Amount/Conc.
bovine serum albumin (BSA)	100 µg/ml
sodium selenite	40 ng/ml
putrescine	16 µg/ml
25 thyroxine	40 ng/ml
tri-iodothyronine	40 ng/ml
transferrin	100 µg/ml
progesterone	40 ng/ml
pyruvate	1 mM
30 glutamine	1 mM
N-Acetyl-L-cysteine	0.06 mg/ml

30

**F. MTT Survival Assay**

- The MTT survival assay was performed as described by Mosmann (1983). MTT
- 35 (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) was dissolved in PBS at 5 mg/ml and sterilized by passage through a 0.22 µm Millipore filter (VWR

Scientific Corp., Westchester, PA). This stock solution was added to the culture well (1:9) and incubated at 37°C for 1 hour. Viable cells with active mitochondria cleaved the tetrazolium ring of MTT into a visible dark blue formazan reaction product. The viable and dead cells in each well were counted by bright-field microscopy.

5

#### G. Immunofluorescence Staining

Cells were fixed with 4% paraformaldehyde for 5 minutes at room temperature. Non-specific binding was blocked by a 30 minute incubation in 50% goat serum containing 1% BSA and 100 mM l-lysine. The cells were then surface-stained with A2B5 antibody (supernatant diluted 1:1) followed by fluorescein-coupled goat anti-mouse IgM (u chain specific, Jackson; 10 µg/ml).

In order to stain intracellular antigens, cells were permeabilized by adding "TRITON" X-100 (0.4%) to the goat serum solution. Cells were stained with mouse monoclonal anti-p53 antibody (pAb240, cat #OP29, Oncogene Science, Uniondale, NY) used at 5 µg/ml. Anti-p53 antibodies were detected with fluorescein-conjugated goat anti-mouse IgG antibody (Jackson Labs).

The coverslips were mounted in "CITIFLOUR" (University of London, Canterbury, England) on glass slides, sealed with nail varnish and examined in a Zeiss Axioscope fluorescence microscope (Carl Zeiss Inc., Thornwood, NY). Oligodendrocyte precursor cells were identified by A2B5 antibody staining. Adult and perinatal oligodendrocyte precursor cells were distinguished based on the pattern of anti-p53 staining: nuclear for adult, cytoplasmic for perinatal.

### EXAMPLE 1

#### 25 Purification and Culture of Adult Oligodendrocyte Precursor Cells

Adult oligodendrocyte precursor cells were purified from the optic nerves of P8 S/D rats as follows.

#### A. Preparation of Panning Plates.

30 Two sets of panning plates, either 100 mm × 15 mm or 150 mm × 15 mm plastic petri dishes (Fisher Scientific, Pittsburgh, PA; Cat No. 8-757-12), were prepared as described below. Dishes made of tissue culture plastic were not used due to potential problems with non-specific cell sticking. None of the incubation solutions used to coat

panning plates were sterilized with 0.22  $\mu$ m filters, since much of the protein would have been lost in the filter.

Panning plates comprising the first set ("negative-selection" plates) were incubated with 5 ml of 50 mM Tris buffer (pH 9.5) containing 5  $\mu$ g/ml affinity-purified goat anti-mouse IgM (mu-chain specific; Accurate Chemical & Scientific Corp., Westbury, NY) for 12 hours at 4°C. The supernatant was removed and the dishes were washed three times with 8 ml of PBS.

The dishes were then incubated with 5 ml of a supernatant from mouse monoclonal cell line OX-7 (Serotec, Oxford, United Kingdom), for at least one hour at room temperature. The supernatant was removed and the plate washed three times with PBS. In order to prevent nonspecific binding of cells to the panning dish, 5 ml of Minimal Essential Medium (MEM; Gibco/BRL) with 2 mg/ml BSA was placed on the plate for at least 20 minutes.

The second set of panning plates ("positive-selection" plates) was incubated with affinity-purified goat anti-mouse IgM (mu-chain specific, Accurate Chemical & Scientific Corp., Westbury, NY), as above, washed, and further incubated with A2B5 monoclonal IgM ascites (ATCC, Accession # CRL 1520) at 1:2000 (Eisenbarth, *et al.*, 1979). The antibodies were diluted in Hepes-buffered Minimal Eagle's Medium (MEM/Hepes, Gibco/BRL) containing bovine serum albumin (BSA, 1mg/ml; Sigma A4161), in order to block the non-specific adherence of cells to the panning plates. The antibody solution was removed, the plates washed three times with PBS, and PBS left on the plates until use.

#### B. Panning Procedure.

The panning procedure is summarized schematically in Figures 1A-1E. An optic nerve cell suspension (20) prepared as above and containing adult oligodendrocyte precursor cells (22), macrophages (24) and various other cells, including Thy1 positive cells (26) and Thy1 negative cells (28), was incubated on a (first set) panning plate (30; 150 mm) derivatized with goat-anti-mouse IgM (31) and OX-7 monoclonal antibody (32) at room temperature for 45 minutes (Fig. 1B). The plate was gently swirled after 20 minutes to ensure access of all cells to the surface of the plate. If cells from more than 10 optic nerves were panned, the nonadherent cells were transferred to another 150-mm anti-mouse IgG/OX-7 panning plate for an additional 30 minutes.

Non-adherent cells were removed with the suspension, filtered through a UV-sterilized 15 micron "NITEX" mesh (Tetko, Elmsford, NY) to remove small clumps of cells, placed on a second set panning plate (34) derivatized with goat-anti-mouse IgM (36) and mouse A2B5 monoclonal IgM (38), and incubated on the plate (Fig. 1C) as described above for 1 hour.

Non-adherent cells were discarded. The plates were washed 8 times with 6 ml of PBS or MEM/Hepes with moderately vigorous agitation to remove all antigen-negative non-adherent cells. When solutions were removed from the panning dishes during washes, they were immediately replaced with fresh solution so that the cells did not dry out.

The progress of nonadherent cell removal was monitored under an inverted phase-contrast microscope, and washing was terminated when only adherent cells remained.

#### C. Removing Adherent Cells from the Plate

Four ml of a trypsin solution (0.125%) were prepared by diluting a trypsin 20X stock (Sigma) into EBSS. Cells adhering to the panning dish (34) were incubated with this solution for 10 minutes in a 5% CO<sub>2</sub> incubator at 37°C. The cells were dislodged by gently pipetting trypsin solution around the plate. Ten ml of a 25% fetal calf serum (FCS; Gibco/BRL) solution were added to inactivate the trypsin and the cells (Fig. 1E) were spun and collected as above. To eliminate traces of FCS, the cells were resuspended and spun down again in an MEM solution containing BSA (0.5%). The cells were then resuspended in MBS culture medium for use in the experiments.

### EXAMPLE 2

#### Proliferative Behavior of Adult v. Perinatal Oligodendrocyte Precursor Cells

The proliferative behavior of adult precursor cells is studied to determine if they divide in response to the same mitogens that elicit perinatal oligodendrocyte precursor cell clonal expansion, and how the cell-cycle time of the adult cells compares with that of the perinatal cells.

Cell proliferation is assayed in two ways. In each experiment, the ability of single factors or combinations of factors (particularly, PDGF+NT-3+IGF-1 and PDGF+bFGF+IGF-1) to induce DNA synthesis is assessed by measuring BrdU incorporation immunohistochemically. In addition, cell division itself is followed by clonal analysis. Cell-cycle time can be approximated by the rate of increase of the average

number of cells per clone over time, when control experiments show that the survival in the study conditions is high.

The proliferation-inducing potential of trophic factors that do not induce perinatal oligodendrocyte precursor cells to divide, such as TGF-alpha, NGF, BDNF, IGF-1, insulin, CNTF, LIF, IL-6, GGF and SCF, is also assessed. The effects of combinations of multiple factors on the division of the adult precursor cells are also examined. The rate of proliferation of adult oligodendrocyte precursor cells *in vitro* and *in vivo* is compared.

To determine whether the adult precursor cells in the adult optic nerve *in vivo* are totally quiescent or whether they are dividing at very slow rates, perhaps with cell cycle times of 5 to 10 days as has been observed in experiments performed in support of the present invention *in vitro*, adult rats are injected with BrdU intraperitoneally one hour prior to purifying the adult precursor cells. The percentage of the purified adult precursor cells that were in DNA synthesis at the time of the injection is determined immunohistochemically.

### EXAMPLE 3

#### Capacity of Adult Precursor Cells to Revert to Perinatal Precursor Cells

The potential of adult oligodendrocyte precursor cells to promote remyelination is improved if they can be induced to revert to perinatal precursor cells, in order to rapidly generate new oligodendrocytes.

Various extracellular signals are evaluated for an effect that could cause the purified adult precursor cells to revert to cells with the behavior and antigenic phenotype of perinatal precursor cells.

The experiments are performed using clonal cultures, as described above. The extracellular signals that are tested include unmyelinated retinal ganglion cells and their axons, known factors that might be released by unmyelinated axons including GGF, FGF and glutamate, perinatal optic nerve extract, and co-culture with activated macrophages that would be expected to be present after injury.

### EXAMPLE 4

#### Remyelination by Adult Precursor Cells

Purified adult and perinatal precursor cells are assayed for their ability to generate oligodendrocytes at sufficient number to support remyelination when transplanted into a developing spinal cord that lacks myelin.



The ability of transplanted glial cells, including purified perinatal oligodendrocyte precursor cells, to myelinate unmyelinated or demyelinated axons has been demonstrated (Utzschneider, *et al.*, 1994). A similar protocol is used, but with purified oligodendrocyte precursor cells instead of mixed glial cells:

- 5 Purified perinatal or adult precursor cells are transplanted into the spinal cord of postnatal day 3 md (myelin-deficient) rats, a mutant rat that lacks myelin (Duncan, *et al.*, 1988; Utzschneider, *et al.*, 1994). A long-lived strain of md rats that lives up to 90 days is preferably employed, so that survival considerations are not limiting.

- 20,000 purified cells resuspended into 1  $\mu$ l PBS are injected via a glass micropipet  
10 into two or three sites along the dorsal columns of the spinal cord. Each recipient rat is anesthetized with fluothane and undergoes a dorsal laminectomy at the thoracolumbar junction. The transplant sites are marked with sterile charcoal before closing the incision. After 2 to 3 weeks, the animals are sacrificed by inhalation anesthesia using fluothane, the spinal cords removed and immersed in glutaraldehyde.

- 15 The spinal cords are further processed with Epon embedding, preparation of 1  $\mu$ m thick semi-thin sections and toluidine staining for viewing with a light microscope. The percentage of axons that have been myelinated after perinatal or adult oligodendrocyte precursor cell transplantation is determined and compared.

20

#### EXAMPLE 5

##### Alternate Method of Purifying Human Adult Oligodendrocyte Precursor Cells

- Human brain tissue is obtained using obtained using standard biopsy (*e.g.*, Wen, *et al.*, 1993) or surgical procedures (*e.g.*, Son, *et al.*, 1994; Shih, *et al.*, 1994; Benbadis,  
25 1995). The tissue is preferably obtained from a region of the brain whose removal results in little or no disruption of the individual's mental functioning (*e.g.*, the right temporal lobe).

- The tissue is prepared, and adult oligodendrocyte precursor cells are purified as described in Example 1, above, except as follows. The first "depletion" plate is derivatized  
30 using an antibody specifically immunoreactive with proteolipid protein (PLP), a major component of the myelin protein in the peripheral nervous system (PNS) and CNS (Meyer-Franke and Barres, 1994). PLP is highly conserved among the vertebrates (Yoshida and Coleman, 1996). Accordingly, antibodies prepared against any mammalian PLP typically cross-react with human PLP. A number of antibodies to the protein have been generated

(see, *e.g.*, Yamamura, *et al.*, 1991). Additionally, antibodies may be generated as described above.

The second panning plate (the "positive selection plate") is derivatized with an antibody specifically immunoreactive with O4. Such antibodies have been generated (*e.g.*,  
5 Sommer and Schachner, 1981, 1982; Gogate, *et al.*, 1994), and may be generated using standard methods (*e.g.*, the methods in the cited publications and/or as described above). Additionally, anti-O4 antibodies specifically immunoreactive with human O4 are commercially available (*e.g.*, from Boehringer-Mannheim, Indianapolis, IN, under catalog No. 1 518 925).

10 Adult oligodendrocyte precursor cells purified as described above can be used immediately, or can be maintained in culture under conditions which support cell division and inhibit differentiation as described herein.

While the invention has been described with reference to specific methods and  
15 embodiments, it is appreciated that various modifications and changes may be made without departing from the invention.

## IT IS CLAIMED:

1. A culture of mammalian cells, where more than about 95% of the cells are adult oligodendrocyte precursor cells.
2. The culture of claim 1, where more than about 99% of the mammalian cells are adult oligodendrocyte precursor cells.
3. The culture of claim 1, where the mammalian cells are derived from optic nerve.
4. The culture of claim 1, where the mammalian cells are derived from temporal lobe.
5. The culture of claim 1, where the mammalian cells are rat cells.
6. The culture of claim 1, where the mammalian cells are human cells.
7. A method of purifying adult oligodendrocyte precursor cells from a suspension of cells derived from adult central nervous system (CNS) tissue, comprising contacting the suspension with a first petri plastic surface derivatized to contain an antibody specifically immunoreactive with Thy-1, wherein a first portion of the cells in the suspension becomes immobilized on said first surface and a second portion of the cells remains non-adherent, and contacting said non-adherent portion of the cells with a second petri plastic surface derivatized to contain a moiety capable of selectively binding a marker preferentially-expressed on oligodendrocyte precursor cells, wherein cells adhering to said second surface are substantially-purified adult oligodendrocyte precursor cells.
8. The method of claim 7, wherein said moiety is the A2B5 antibody.
9. The method of claim 7, wherein said moiety is the NG-2 monoclonal antibody.

10. The method of claim 7, wherein said moiety is peanut agglutinin.

11. An isolated population of adult oligodendrocyte precursor cells, where more than about 95% of the population are adult oligodendrocyte precursor cells.

5

12. The population of claim 11, where more than about 99% of the population are adult oligodendrocyte precursor cells.

13. The population of claim 11, where the cells are human cells.

10

14. A pharmaceutical composition, comprising  
an isolated population of adult oligodendrocyte precursor cells, where more than about 95% of the population are adult oligodendrocyte precursor cells.

15

15. The composition of claim 14, where more than about 99% of the population are adult oligodendrocyte precursor cells.

16. The composition of claim 15, where the cells are purified from optic nerve.

20

17. The composition of claim 15, where the cells are purified from rat.

18. The composition of claim 14, for use in a treatment of an individual suffering from symptoms due to a demyelinating disease or condition, said treatment comprising delivering said composition to a demyelinated region of the individual's central nervous  
25 system, wherein said region is responsible for causing at least a portion of said symptoms.

19. The composition of claim 18, wherein said demyelinating disease or condition is multiple sclerosis.

30

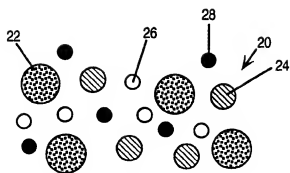
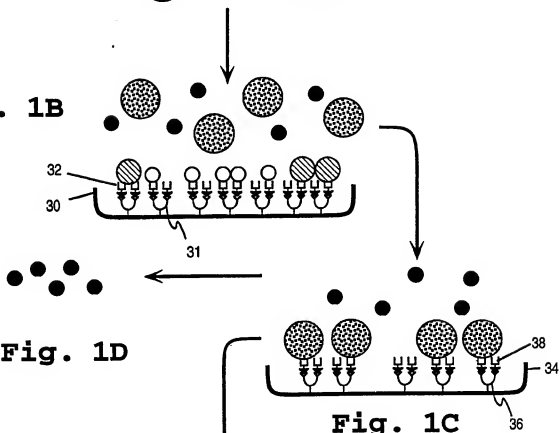
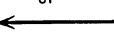
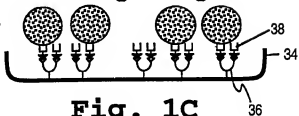
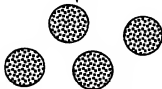
20. The composition of claim 18, wherein said composition is autologous to said individual.

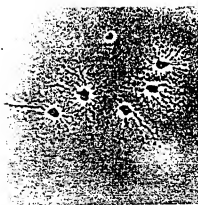
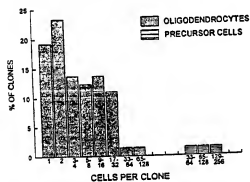
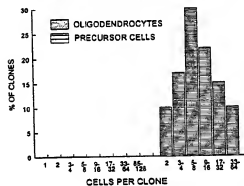
21. A method of culturing adult oligodendrocyte precursor cells, comprising incubating said cells in a medium which

- (i) is substantially free of feeder cells or medium conditioned by feeder cells,
- (ii) is substantially serum-free, and
- (iii) contains N-Acetyl-L-cysteine, PDGF and NT-3,

wherein said culturing results in maintenance of said adult oligodendrocyte

5 precursor cells in an undifferentiated state.

**Fig. 1A****Fig. 1B****Fig. 1D****Fig. 1C****Fig. 1E**

**Fig. 2A****Fig. 2B****A. Perinatal Oligodendrocyte Precursor Cells, 12 days in vitro****Fig. 3A****B. Adult Oligodendrocyte Precursor Cells, 30 days in vitro****Fig. 3B****Fig. 4A****Fig. 4B**

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/13279

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C12N 5/00, 5/02; A01N 63/00

US CL : 435/240.2, 240.21, 240.25; 424/93.1, 93.21

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.2, 240.21, 240.25; 424/93.1, 93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, JAPOABS, MEDLINE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GARD et al. Oligodendroblasts distinguished from O-2A glial progenitors by surface phenotype (O4 + GalC-) and response to cytokines using signal transducer LIFR beta. Developmental Biology, February 1995, Vol. 167, No. 2, pages 596-608, see Abstract.	1-21
Y	WHITTEMORE et al. Concurrent isolation and characterization of oligodendrocytes, microglia and astrocytes from adult human spinal cord. International Journal of Development Neuroscience, December 1993, Vol. 11, No. 6, pages 755-764, see Abstract.	1-21

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A document defining the general state of the art which is not considered to be of particular relevance	*X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E earlier document published on or after the international filing date	*Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G document member of the same patent family
*O document referring to an oral disclosure, use, exhibition or other means	
*P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

12 NOVEMBER 1996

Date of mailing of the international search report

03 DEC 1996

Name and mailing address of the ISA/US

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/13279

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HART et al. PDGF and intracellular signaling in the timing of oligodendrocyte differentiation. Journal Cell Biology, December 1989, Vol. 109, pages 3411-3417, see Abstract.	1-17
Y	Juurlink et al. Hyperthermic injury of oligodendrocyte precursor cells: implications for dysmyelination disorders. Brain Research, April 1994, Vol. 641, No. 2, pages 353-356, see Abstract.	1-15
Y	Armstrong et al. Pre-oligodendrocytes from adult human CNS. Journal of Neuroscience, April 1992, Vol. 12, No. 4, pages 1538-1547, see Abstract.	1-21
Y	US 5,219,837 A (COHEN et al.) 15 June 1993, see entire document.	1-21